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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:	Group Art Unit: 1632
Sylvie ROUX, et al.	
Application No.: 10/662,808	Examiner: Shin Lin CHEN
Filed: September 16, 2003	Confirmation No.: 2497
For: *IN VIVO* MODULATION OF NEURONAL TRANSPORT	

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Sir:

APPEAL BRIEF UNDER BOARD RULE § 41.37

In support of the Notice of Appeal filed June 20, 2007, and further to Board Rule 41.37, Appellant presents this brief and encloses herewith a check for the fee of $500.00, as required under 37 C.F.R. § 1.17(c). This brief is being filed concurrently with a Petition for Extension of Time of five months and the fee of $2230.

This Appeal responds to the December 21, 2006, Final Rejection of claims 32-33 and 68-73, all of which are set forth in the attached Appendix A. If any additional fees are required or if the enclosed payment is insufficient, Appellant requests that the required fees be charged to Deposit Account No. 06-0916.

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I. Real Party in Interest

The assignment recorded on May 3, 2004, at reel 015300, frame 0457 designates Institut Pasteur and Centre Nationale De La Recherché Scientifique as the Assignees of record. Institut Pasteur and Centre Nationale De La Recherché Scientifique are, therefore, the real parties in interest in this appeal.

II. Related Appeals and Interferences

There are currently no other appeals or interferences, of which Appellant, Appellant's legal representative, or Assignee are aware, that will directly affect, be directly affected by, or have a bearing on the Board's decision in the pending appeal.

III. Claim Status

Claims 32-33 and 68-73 are pending and stand presently rejected. [Appendix A]. Appellant now appeals the rejection of claims 32-33 and 68-73.

IV. Status of Amendments

The most recent claim amendments were filed with Appellant's Response After Nonfinal Action of October 2, 2006, and entered by the Final Rejection of December 21, 2006.

V. Summary of Claimed Subject Matter

Nerve cells, or neurons, transmit information to other neurons and to muscle cells via chemical neurotransmitters. A nerve cell synthesizes neurotransmitters in the central region of the cell (the cell body) then transports them along cellular structures called axons to their site of action, where they are released from the nerve cell. This directional movement from the site of synthesis in the neuronal cell body to the site of action at the cell periphery, is called anterograde transport.

Neurons also transport cellular contents from the periphery back toward the cell body. This process is called retrograde transport.

Tetanus toxin, a nerve cell poison, takes advantage of the ability of the nerve cell to perform retrograde transport. Specifically, tetanus toxin gains entry into the nervous system via retrograde transport. Tetanus toxin present in muscle enters the nervous system through synapses of motor neurons (also called first order neurons) at the neuromuscular junction. It enters the neuron via endocytosis, and travels via retrograde transport to the cell body of that neuron. The toxin continues traveling through the nervous system by passing through synapses connecting the motor neurons with second order neurons and moving up the axon of the second order neuron, via retrograde transport, to the cell body. Transport continues across synapses, through a chain of interconnected neurons, into the central nervous system. [Exhibit 1 at page 9400].

Tetanus toxin poisons neurons by preventing them from releasing certain neurotransmitters that convey information to neighboring neurons. The intact toxin can be selectively cleaved by a protease to form a fragment known as tetanus toxin fragment C ("TTC"). No longer toxic, TTC is still capable of uptake into, and retrograde transport by, the neuron. [Specification at ¶12; ¶16].

The inventors have discovered that TTC can function as a carrier for the delivery of biologically active agents, for example therapeutics, to the central nervous system. [Specification at ¶16]. TTC, attached to a visualizable reporter molecule and injected into muscle, enters motor neurons through synapses at the neuromuscular junction. [Specification at ¶¶78-87; ¶¶95-106, Figure 6]. The labeled TTC proceeds, via

retrograde transport, into higher order neurons, including those in the brainstem. [Specification at ¶¶68-70; ¶¶107-112].

The differentiation, growth, and survival of neurons depends on neurotrophic proteins, which are expressed throughout the nervous system and play diverse roles in shaping and modulating connections between neurons. Neurotrophins are a group of structurally and functionally related proteins, members of the neurotrophin family include brain derived neurotrophic factor (BDNF), neurotrophin 4 (NT-4), and glial-derived neurotrophic factor (GDNF). Neurotrophins were known to bind to, activate, and exert certain of their physiological effects through the receptors TrkB and/or cRET. [Specification at ¶¶26-29].

The inventors have also discovered that certain members of the neurotrophin family modulate the retrograde transport of tetanus toxin. [Specification at ¶24]. BDNF, NT-4, and GDNF influenced the neuronal localization and internalization of tetanus toxin. [Specification at ¶15]. A fusion protein of TTC and the reporter protein green fluorescent protein ("GFP") was shown to be rapidly localized to the neuromuscular junction, under the influence of these neurotrophins. [Specification at ¶116; ¶118; ¶120]. The neurotrophin effect was shown to be dose-dependent and specific for some (BDNF, NT-4, and GDNF), but not other, members of the neurotrophin family. [Specification at ¶¶125-127; ¶¶128-129; Table 2; Figure 7].

Neurotrophins modulate TTC uptake via a receptor-independent mechanism, enhancing the uptake of an uncoated vesicular compartment, which is distinct from the synaptic vesicle compartment. [Specification at ¶¶130-131; Figure 8; ¶¶132-135; Figure 9]. This mechanism was distinguished from the mechanism cholera toxin uses to enter

neurons by comparing the lipid microdomains each toxin used to bind to and enter the neuron. [Specification at ¶¶136-139; Figure 10; Figure 11].

The claims now appealed recite methods of modulating the neuronal transport of tetanus toxin and its C fragment with the neurotrophins BDNF, NT-4, and/or GDNF.

VI. Appealed Claims

A. Identification of Claims with Reference to the Specification

Claim No.	Claim	Corresponding Disclosure
32	A method of modulating the transport in a neuron of a tetanus toxin or a fusion protein comprising a fragment C of the tetanus toxin, wherein the method comprises administering to the neuron a Brain Derived Neurotrophic Factor (BDNF), a Neurotrophin 4 (NT-4), or Glial-Derived Neurotrophic Factor (GDNF) in an amount sufficient to thereby modulate the neuronal transport of the tetanus toxin or the fusion protein	¶4 ¶12 ¶24 ¶¶104-129 Figure 6 Figure 7 Table 2 ¶¶130-135 Figure 9
33	wherein Brain Derived Neurotrophic Factor (BDNF), Neurotrophin 4 (NT-4), or Glial-Derived Neurotrophic Factor (GDNF) thereby increases internalization of the tetanus toxin or fusion protein at a neuromuscular junction	¶¶125-129 Figure 6 Figure 7 Table 2
68	wherein the tetanus toxin is administered with Brain Derived Neurotrophic Factor (BDNF)	¶31
69	wherein the tetanus toxin is administered with Neurotrophin 4 (NT-4)	¶31
70	wherein the tetanus toxin is administered with Glial-Derived Neurotrophic Factor (GDNF)	¶31 original claim 55
71	wherein the fusion protein comprising a fragment C of the tetanus toxin is administered with Brain Derived Neurotrophic Factor (BDNF)	¶31
72	wherein the fusion protein comprising a fragment C of the tetanus toxin is administered with	¶¶31

	Neurotrophin 4 (NT-4)	
73	wherein the fusion protein comprising a fragment C of the tetanus toxin is administered with Glial-Derived Neurotrophic Factor (GDNF)	¶¶31 original claim 55

B. Appellant's Claim Interpretation

1. Claim 32: Modulating Neuronal Transport

Claim 32 recites a method of modulating the neuronal transport of a toxin by administering a neurotrophic factor to the neuron. The toxin can be either intact tetanus toxin or a fusion protein comprising a fragment of the tetanus toxin. Brain Derived Neurotrophic Factor (BDNF), Neurotrophin 4 (NT-4), and Glial-Derived Neurotrophic Factor (GDNF), when administered to the neuron in a sufficient amount, modulate the transport of the toxin. [Specification at ¶24].

By "transport in a neuron," Appellant means the retrograde neuronal transport of tetanus toxin, TTC, or a fusion protein comprising TTC. This transport includes recruitment to the neuromuscular junction, internalization into a neuron via endocytosis from the muscle, and transport toward the neuronal cell body in membrane bound vesicles. [Exhibit 1 at page 9400; specification at ¶¶68-70; ¶¶100-112]. Neuronal transport also encompasses retrograde and transsynaptic trafficking of the toxin from one neuron to another in the direction of the central nervous system, including neurons within the central nervous system. [Specification at ¶104-112]. Neuronal transport may occur *in vitro* or *in vivo*. [Specification at ¶¶100-112; ¶¶104-112].

By "modulate," Appellant refers generally to the ability of a compound to act as an agonist or an antagonist of a certain reaction or activity, thus encompassing both increasing and decreasing the activity. [Specification at ¶25]. In the context of the

appealed claims, "modulation" refers to an increase in the neuronal transport of tetanus toxin, TTC, or a fusion protein comprising TTC. [Specification at Table 2].

By "in an amount sufficient to thereby modulate neuronal transport," Appellant means the amount of neurotrophin needed to increase transport of tetanus toxin, TTC, or a fusion protein comprising TTC above the level of transport occurring in the absence of neurotrophin. This amount can be determined experimentally, for example, as described in Examples 10 and 11 of the specification. [Specification at ¶¶125-129]. Increasing concentrations of neurotrophin were injected into either the *Levator auris longus* or the gastrocnemius muscle, resulting in a dose-dependent increase in the level of toxin at the neuromuscular junction up to a maximum concentration, after which the level of toxin began to decrease. [*Id.*].

By "tetanus toxin," Appellant means the toxin produced by *Clostridium tetani* as an inactive, single polypeptide chain, as well as the toxin activated by selective proteolytic cleavage to a heavy chain and a light chain, joined by disulfide linkage. [Specification at ¶3]. By "tetanus toxin," Appellant also refers to a fragment comprising or encoding substantially all of the 451 amino acids at the carboxyl terminus of the tetanus toxin heavy chain ("TTC"). [Specification at ¶5; ¶61]. The term "tetanus toxin" further encompasses a fusion protein comprising substantially all of TTC and at least one other molecule having biological function. For example, the fusion protein may comprise a reporter gene or polypeptide, such as *LacZ*, β-galactosidase, or green fluorescent protein ("GFP"). [Specification at ¶12; ¶62]. A "tetanus toxin" may also comprise a therapeutic gene or polypeptide, such as a neurotrophic factor, growth factor, or antibody, by way of non-limiting example. [Specification at ¶62].

2. Claim 33: Internalization at the Neuromuscular Junction

Tetanus toxin present in the muscle first enters the neuron at the neuromuscular junction, the site where a motor neuron innervates the muscle to regulate its contraction. [Specification at ¶60]. The toxin enters the presynaptic motor nerve terminal, the structure of the neuromuscular junction responsible for secreting neurotransmitters in the vicinity of the muscle. [Specification at ¶124]. BDNF, NT-4, and GDNF increase the amount of tetanus toxin present at the neuromuscular junction. [Specification at ¶125-129; Table 2]. The motor neuron internalizes the toxin by endocytosis and the endocytic vesicles containing the toxin move through the neuron in a retrograde direction, from the synapse to the axon. [Specification at ¶126; Figure 7; ¶134; Figure 9].

3. Claims 68-73: Administered with a Neurotrophic Factor

The neurotrophic factor BDNF, NT-4, and/or GDNF can be administered to neurons prior to, concurrently with, or following the entry of tetanus toxin into the neuron. [Specification at ¶31].

VII. Grounds of Rejection to Be Reviewed on Appeal, Under 35 U.S.C. § 112, ¶2: The Claims Allegedly Omit an Essential Step

The Examiner rejected all of the pending claims, under 35 U.S.C. § 112, ¶2, as "incomplete for omitting essential steps," citing the M.P.E.P. as the source of authority for the rejection. [Office Action of December 21, 2006, at page 2]. "[A] claim which fails to interrelate essential elements of the invention as defined by applicant(s) in the specification may be rejected under 35 U.S.C. 112, second paragraph, for failure to point out and distinctly claim the invention." [M.P.E.P. § 2172.01].

According to the Examiner, the omitted steps are "how to modulate the neuronal transport of the tetanus toxin or the fusion protein" and "whether the neuronal transport is modulated." [Office Action of December 21, 2006, at page 2]. The Examiner then states that the method step refers to administering a neurotrophin but "fails to refer back to the preamble of the claimed method, i.e. modulating the transport in the neuron. [*Id.*]. Appellant previously addressed this ground for rejection by amending claim 32 to recite that the neurotrophin is administered "in an amount sufficient to thereby modulate the neuronal transport of the tetanus toxin or the fusion protein." [Amendment and Response to the Office Action of June 1, 2006, filed October 2, 2006, at page 6].

The Examiner did not consider Appellant's amendment sufficient to overcome the rejection and reasoned that, with respect to claims 32 and 33, "it remains unclear how and when the tetanus toxin . . . is added to the neuron" and, with respect to all pending claims, "it is also unclear how to modulate the neuronal transport of the tetanus toxin or the fusion protein, i.e. how to detect the presence of tetanus toxin in the neuron." [Office Action of December 21, 2006, at pages 2-3].

VIII. Argument

The second paragraph of 35 U.S.C. § 112 requires that two separate conditions are met. First, the claims must set forth the subject matter that the applicants regard as their invention; and second, the claims must particularly point out and distinctly define the metes and bounds of the subject matter that will be protected by the patent grant. The first requirement is a subjective one because it is dependent on what the applicants regard as the invention. The second requirement is an objective one because it is not dependent on the views of the applicant or any particular individual, but is evaluated in the context of whether the claim is definite — i.e., whether the scope of the claim is

clear to a hypothetical person possessing the ordinary level of skill in the pertinent art. [M.P.E.P. § 2173.02].

With regard to the first requirement, there is no evidence of record that Appellant is claiming anything other than what it regards as the invention. Thus, the claims must be deemed to be in compliance with this requirement.

The claims fulfill the second requirement because they are sufficiently clear and precise to convey the scope of the claimed invention to one of ordinary skill in the art. Claim definiteness is analyzed "not in a vacuum, but always in light of the teachings of the prior art and of the particular application disclosure as it would be interpreted by one possessing the ordinary level of skill in the pertinent art." [*Energizer Holdings, Inc. v. International Trade Com'n*, 435 F.3d 1366, 1370, 77 U.S.P.Q.2d (BNA) 1625, 1628 (Fed. Cir. 1996)]. As discussed above, and as is further made evident by examination of the specification and the art presented as Exhibit 1, one of ordinary skill would recognize that the claim scope is defined as providing a method of modulating the neuronal transport of tetanus toxin by administering a sufficient amount of BDNF, NT-4, and/or GDNF.

Claim 32 relates the method step to the preamble. In fact, Appellant amended claim 32 for the purpose of relating the method of modulating neuronal transport to the preamble reciting the administration of the neurotrophic factor. [Amendment in response to the Office Action of June 21, 2007]. The preamble of claim 32 recites "[a] method of modulating the transport in a neuron of a tetanus toxin or a fusion protein comprising a fragment C of the tetanus toxin." The claim then states that the method comprises administering one of three neurotrophic factors to the neuron, and concludes

by relating the administration of the neurotrophic factor to the preamble by reciting "in an amount sufficient to thereby modulate the neuronal transport of the tetanus toxin or the fusion protein." [Response to the Office Action of June 21, 2007 (emphasis added)].

The claims, read in light of the prior art and the disclosure, clearly and precisely convey how and when the toxin is added to the neuron. The addition of tetanus toxin to neurons is well-known in the art. [Specification at ¶4; ¶¶6-10]. The mechanics of how the toxin is added are disclosed in clear detail in the specification. For example, "the tongue muscle was injected using an Hamilton syringe (20 µl per animal) while under general anesthesia with 3% Avertin (15 µl/g of animal). The protein concentration was 0.5 to 5 µg/µl in PBS; therefore, mice received approximately 10 to 100 µg per injection." [Specification at ¶96].

The specification also clearly and precisely defines the timing of when the toxin is added. The modulator "can be administered to neuronal cells that already contain a tetanus toxin or a fusion protein. Alternatively, the tetanus toxin or fusion protein can be administered concurrently with or after the administration of the agonist or antagonist." [Specification at ¶31].

The Examiner's conflation of modulating transport with detecting the modulated protein appears to reflect a fundamental misunderstanding of the claimed invention. "[I]t is also unclear how to modulate the neuronal transport of the tetanus toxin or the fusion protein, i.e., how to detect the presence of tetanus toxin in the neuron." [Office Action of December 21, 2006, at page 2, emphasis added]. The American Heritage College Dictionary defines "i.e.," as the abbreviation for the Latin phrase id est, or "that is."

[Exhibit 3]. The Examiner has thus stated that it is unclear how to modulate neuronal transport, that is how to detect the toxin in the neuron.

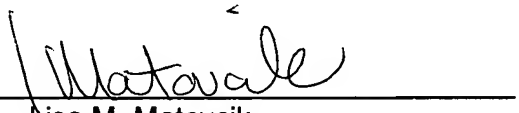
"Modulating neuronal transport" is clearly and precisely understood by one of skill in the art, in view of the prior art and the invention disclosure, as described in detail above. Modulating neuronal transport refers to an increase in the retrograde neuronal transport of tetanus toxin. [Exhibit 1 at page 9400; specification at ¶4; ¶12; ¶24; ¶104-129; Figure 6; Figure 7; Table 2; ¶¶130-135; Figure 9]. Detecting the presence of the toxin is quite different from modulating transport; the latter involves visualizing or otherwise tracking the toxin. The claims are directed to modulating neuronal transport and not to detecting the transported toxin. The claims clearly and precisely set forth the metes and bounds of Appellant's method of modulating the neuronal transport of tetanus toxin.

IX. Conclusion

For the reasons given above, Appellant respectfully requests that the Board reverse the rejection of claims 32-33 and 68-73.

FINNEGAN, HENDERSON, FARABOW,
GARRETT & DUNNER, L.L.P.

Dated: December 19, 2007

By: 

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APPENDIX A

Claims Appendix to Appeal Brief Under Rule 41.37(c)(1)(viii)

The following list of claims reflects the most recent amendment of the claims, filed October 2, 2006 and entered December 21, 2006.

32. A method of modulating the transport in a neuron of a tetanus toxin or a fusion protein comprising a fragment C of the tetanus toxin, wherein the method comprises administering to the neuron a Brain Derived Neurotrophic Factor (BDNF), a Neurotrophin 4 (NT-4), or Glial-Derived Neurotrophic Factor (GDNF) in an amount sufficient to thereby modulate the neuronal transport of the tetanus toxin or the fusion protein.

33. The method according to claim 32, wherein Brain Derived Neurotrophic Factor (BDNF), Neurotrophin 4 (NT-4), or Glial-Derived Neurotrophic Factor (GDNF) thereby increases internalization of the tetanus toxin or fusion protein at a neuromuscular junction.

68. The method according to claim 32, wherein the tetanus toxin is administered with Brain Derived Neurotrophic Factor (BDNF).

69. The method according to claim 32, wherein the tetanus toxin is administered with Neurotrophin 4 (NT-4).

70. The method according to claim 32, wherein the tetanus toxin is administered with Glial-Derived Neurotrophic Factor (GDNF).

71. The method according to claim 32, wherein the fusion protein comprising a fragment C of the tetanus toxin is administered with Brain Derived Neurotrophic Factor (BDNF).

72. The method according to claim 32, wherein the fusion protein comprising a fragment C of the tetanus toxin is administered with Neurotrophin 4 (NT-4).

73. The method according to claim 32, wherein the fusion protein comprising a fragment C of the tetanus toxin is administered with Glial-Derived Neurotrophic Factor (GDNF).

APPENDIX B

Evidence Appendix to Appeal Brief Under Rule 41.37(c)(1)(ix)

Exhibit 1: Coen, L. et al., Construction of hybrid proteins that migrate retrogradely and transynaptically into the central nervous system, *Proc. Natl. Acad. Sci., USA*, 94:9400-9405 (1997).

Exhibit 2: *Energizer Holdings, Inc. v. International Trade Com'n*, 435 F.3d 1366, 77 U.S.P.Q.2d (BNA) 1625.

Exhibit 3: American Heritage College Dictionary (3rd ed. 2000) at 675.

Exhibit 1

Proc. Natl. Acad. Sci. USA
Vol. 94, pp. 9400–9405, August 1997
Neurobiology

Construction of hybrid proteins that migrate retrogradely and transynaptically into the central nervous system

(motoneuron diseases/transneuronal transport/retrograde tracer/gene therapy/tetanus toxin C fragment)

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ABSTRACT **The nontoxic proteolytic C fragment of tetanus toxin (TTC peptide) has the same ability to bind nerve cells and be retrogradely transported through a synapse as the native toxin. We have investigated its potential use as an *in vivo* neurotropic carrier. In this work we show that a hybrid protein encoded by the *lacZ*–TTC gene fusion retains the biological functions of both proteins *in vivo*—i.e., retrograde transynaptic transport of the TTC fragment and β-galactosidase enzymatic activity. After intramuscular injection, enzymatic activity could be detected in motoneurons and connected neurons of the brainstem areas. This strategy could be used to deliver a biological activity to neurons from the periphery to the central nervous system. Such a hybrid protein could also be used to map synaptic connections between neural cells.**

Tetanus toxin is a potent neurotoxin of 1,315 amino acids that is produced by *Clostridium tetani* (1, 2). Tetanus toxin prevents the inhibitory neurotransmitter release from spinal cord interneurons by a specific mechanism of cell intoxication (for review, see ref. 3). This pathological mechanism has been demonstrated to involve retrograde axonal and transynaptic transport of the tetanus toxin. The toxin is taken up by nerve endings at the neuromuscular junction but does not act at this site; rather, the toxin is transported into a vesicular compartment and travels along motor axons for a considerable distance until it reaches its targets. The transynaptic movement of tetanus toxin was first demonstrated by autoradiographic localization in spinal cord interneurons after injection into a muscle (4). However, previous studies of transynaptic passage of tetanus toxin from motoneurons were limited by the rapid development of clinical tetanus and death of the experimental animal (4–6).

The C fragment of tetanus toxin obtained by protease digestion, the TTC fragment, has been shown to be transported by neurons in a similar manner to that of the native toxin without causing clinical symptoms (7–10). A recombinant TTC fragment was reported to possess the same properties as the fragment obtained by protease digestion (11). The fact that an atoxic fragment of the toxin molecule was able to migrate retrogradely within the axons and to accumulate into the central nervous system (CNS) led to speculation that such a fragment could be used as a neurotrophic carrier (12). A TTC fragment chemically conjugated to various large proteins was taken up by neurons in tissue culture (13) and by motor neurons in animal models (12, 14, 15). In a more recent *in vitro* study, the human CuZn superoxyde dismutase, SOD-1, fused to the TTC fragment was internalized by neurons and retained some of its biological functions (16).

In this report, we demonstrate that the hybrid protein produced from a *lacZ*–TTC gene fusion can be retrogradely transported across a synapse *in vivo*. The recombinant TTC fragment could therefore be used as an efficient carrier to deliver foreign biological activities from the periphery into neurons of the CNS. This strategy could also be exploited to analyze synaptic activity in neural networks and allow their *in vivo* mapping.

MATERIALS AND METHODS

Plasmid Constructions. *TTC cloning.* Full-length TTC DNA was generated from the genomic DNA from the *Clostridium tetani* strain (a gift from M. Popoff, Pasteur Institute) using PCR. Three overlaping fragments were synthesized: PCR1 of 465 bp (primer 1, 5′-CCC CCC GGG CCA CCA TGG TTT TTT CAA CAC CAA TTC CAT TTT CTT ATT C-3′; and primer 2, 5′-CTA AAC CAG TAA TTT CTG-3′), PCR2 of 648 bp (primer 3, 5′-AAT TAT GGA CTT TAA AAG ATT CCG C-3′; and primer 4, 5′-GGC ATT ATA ACC TAC TCT TAG AAT-3′), and PCR3 of 338 bp (primer 5, 5′-AAT GCC TTT AAT AAT CTT GAT AGA AAT-3′; and primer 6, 5′-CCC CCC GGG CAT ATG TCA TGA ACA TAT CAA TCT GTT TAA TC-3′). The three fragments were sequentially introduced into pBluescript KS+ (Stratagene) to give pBS:TTC plasmid. The upstream primer 1 also contains an optimized eukaryotic ribosome binding site and translational initiation signals. The DNA sequence of all PCR products was identical to that of native TTC DNA (11).

pGEX:lacZ–*TTC:* pGEX:*lacZ* was obtained by cloning a *SmaI*/*XhoI lacZ* fragment from the pGNA vector (a gift from H. Le Mouellic, Pasteur Institute), into pGEX 4T-2 (Pharmacia). PCR was used to convert the *lacZ* stop codon into an *NcoI* restriction site. Two primers (upstream, 5′-CTG AAT ATC GAC GGT TTC CAT ATG-3′; and downstream, 5′-GGC AGT CTC GAG TCT AGA CCA TGG CTT TTT GAC ACC AGA C-3′) were used to amplify the sequence between *NdeI* and *XhoI*, generating pGEX:*lacZ(NcoI)* from pGEX:*lacZ*. pGEX:*lacZ*–TTC was obtained by insertion of the TTC *NcoI*/*XhoI* fragment into pGEX:*lacZ(NcoI)*, fusing TTC immediately downstream of the *lacZ* coding region and in the same reading frame.

Purification of the Hybrid Protein. The *Escherichia coli* strain SR3315 (a gift from A. Pugsley, Pasteur Institute) transfected with pGEX:*lacZ*–TTC was used for protein production. An overnight bacterial culture was diluted 1:100 in Luria–Bertani medium containing 100 μg/ml ampicillin, and grown for several hours at 32°C until an OD of 0.5 was reached. Induction from the Ptac promoter was achieved by the addition of 1 mM isopropyl β-D-thiogalactoside and 1 mM $MgCl_2$ and a further 2 hr of incubation. The induced bacteria were pelleted by centrifugation for 20 min at 3,000 rpm, washed with PBS, and resuspended in lysis buffer containing 0.1 M Tris (pH

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Abbreviations: CNS, central nervous system; TTC, tetanus toxin fragment C; β-gal, β-galactosidase; X-Gal, 5-bromo-4-chloro-3-indolyl β-D-galactoside.

*L.C. and R.O. contributed equally to this work.
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7.8), 0.1 M NaCl, 20% glycerol, 10 mM EDTA, 0.1% Triton X-100, 4 mM DTT, 1 mg/ml lysosyme, and a mixture of anti-proteases (100 μg/ml Pefablok/1 μg/ml leupeptin/1 μg/ml pepstatin/1 mM benzamidine). After cell disruption in a French Press, total bacterial lysate was centrifuged for 10 min at 30,000 rpm. The resulting supernatant was incubated overnight at 4°C with the affinity matrix glutathione-Sepharose 4B (Stratagene) with slow agitation. After centrifugation for 5 min at 3,000 rpm, the matrix was washed three times with the same lysis buffer but without lysosyme and glycerol, and then three times with PBS. The resin was incubated overnight at 4°C with Thrombin (10 units/ml; Sigma) in PBS to cleave the β-galactosidase (β-gal)–TTC fusion protein from the glutathione *S*-transferase sequence and thereby elute it from the affinity column. Concentration of the eluted fusion protein was achieved by centrifugation in centricon X-100 tubes (Amicon; 100,000 molecular weight cutoff membrane).

Purified hybrid protein was analyzed by Western blotting after electrophoretic separation in 8% acrylamide SDS/PAGE under reducing conditions followed by electrophoretic transfer onto nitrocellulose membranes (0.2 mm porosity; Bio-Rad). Immunodetection of blotted proteins was performed with a Vectastain ABC-alkaline phosphatase kit (Vector Laboratories) and diaminobenzidine color development. Antibodies were used as follows: rabbit anti-β-gal antisera (Cappel Laboratories), dilution 1:1,000; rabbit anti-TTC antisera (Calbiochem), dilution 1:20,000. A major band with a relative molecular mass of 180 kDa corresponding to the β-gal–TTC hybrid protein was detected with both anti-β-gal anti-TTC antibodies.

Binding and Internalization of Recombinant Protein in Differentiated 1009 Cells. The 1009 cell line was derived from a spontaneous testicular teratocarcinoma arising in a recombinant inbred mouse strain (129 × B6) (17). The 1009 cells were grown in DMEM containing 10% fetal calf serum and passaged at subconfluence. *In vitro* differentiation with retinoic acid and cAMP was performed as described (18). Eight days after retinoic acid treatment, cells were used for the internalization experiments with either the hybrid protein or β-gal.

Binding and internalization of the β-gal–TTC fusion were assessed using a modified protocol (16). Differentiated 1009 cells were incubated for 2 hr at 37°C with 5 μg/ml of β-gal–TTC or β-gal protein diluted in binding buffer (0.25% sucrose/20 mM Tris acetate/1 mM $CaCl_2$/1 mM $MgCl_2$/0.25% BSA in PBS). The cells were then incubated with 1 μg/ml Pronase E (Sigma) in PBS for 10 min at 37°C, followed by washing with proteases inhibitors diluted in PBS (100 μg/ml Pefablok/1 mM benzamidine).

The cells were fixed with 4% formalin in PBS for 10 min at room temperature and then washed extensively with PBS. β-Gal activity was detected on fixed cells by an overnight staining at 37°C in 5-bromo-4-chloro-3-indolyl β-D-galactoside (X-Gal) solution (0.8 mg/ml X-Gal/4 mM potassium ferricyanide/4 mM potassium ferrocyanide/4 mM $MgCl_2$ in PBS). For electron microscopy, the cells were further fixed in 2,5% glutaraldehyde for 18 hr and then processed as described (19).

For immunohistochemical labeling, cells were fixed with 4% paraformaldehyde in PBS for 10 min at room temperature (RT), then washed extensively with PBS, followed by a 1-hr incubation at RT with 2% BSA/0.02% Triton X-100 in PBS. Cells were coincubated in primary antibodies diluted in 2% BSA/0.02% Triton X-100 in PBS for 2 hr at RT. Antibodies used were a mouse anti-neurofilament antibody (NF, 200 kDa; dilution 1:50; Sigma) or the rabbit anti-TTC antibody (dilution 1:1,000). The labeling was visualized using fluorescent secondary antibodies: Cy3, goat anti-rabbit IgG (dilution 1:500; Amersham) or anti-mouse IgG with extravidin–fluorescein isothiocyanate (dilution 1:200; Sigma). Cells were mounted in moviol and visualized with epifluorescence.

***In Vivo* Recombinant Protein Injections.** Fourteen-week-old B6D2F1 mice were obtained from Iffa-Credo. The animal's tongue muscle was injected with 3% Avertin (15 μl/g of animal) using an Hamilton syringe (20 μl per animal) while under general anesthesia. The protein concentration was 0.5–5 μg/μl in PBS; therefore mice received approximately 10–100 μg per injection. Animals were kept alive for 12–48 hr postinjection to permit migration of the injected protein, and in no case were any tetanus symptoms detected. The mice were killed by intracardiac perfusion with 4% paraformaldehyde in PBS while under deep anesthesia. Brains were harvested, rinsed in PBS, and incubated in 15% sucrose overnight at 4°C, then mounted in tissue-tek before sectioning into 15-μm-thick slices using a cryostat.

Histology, Immunohistology, and X-Gal Staining. For *in toto* X-Gal staining of the dissected brain and tongue, mice (10 animals) were killed and fixed as described above. The brain was further cut with a scapel along a median plane and directly incubated for 12 hr in X-Gal solution.

For immunohistology, sections were incubated in a 1:5,000 dilution of anti-TTC antibody in 2% BSA/0.02% Triton X-100 in PBS overnight at 4°C after nonspecific antibody binding sites were blocked by a 1-hr incubation in the same buffer. Antibody detection was carried out using the Vectastain ABC alkaline phosphatase kit with diaminobenzidine color development. For X-Gal staining, sections were incubated in X-Gal solution and counterstained for 30 sec with hematoxylin [1:5 (vol/vol)] in PBS. Histology on adjacent sections was done after X-Gal staining, using a 30-sec incubation in hematoxylin/thionin solution. All sections were mounted in moviol before light microscopy analysis.

RESULTS

Internalization of the β-Gal–TTC Fusion Protein by Neurons *in Vitro*. Differentiation of 1009 cells with retinoic acid and cAMP *in vitro* yields neuronal and glial cells (18, 20). X-Gal staining or immunolabeling were performed after incubation with the β-gal–TTC fusion protein or with either the β-gal or TTC proteins alone. Only when the hybrid protein was incubated with differentiated 1009 cells was a strong X-Gal staining detected in cells having a neuronal phenotype. No signal was detected when β-gal alone was incubated under the same conditions (Fig. 1 *A* and *B*). A similar X-Gal staining pattern was obtained after pronase treatment of the cells to remove surface bound proteins, indicating that the hybrid

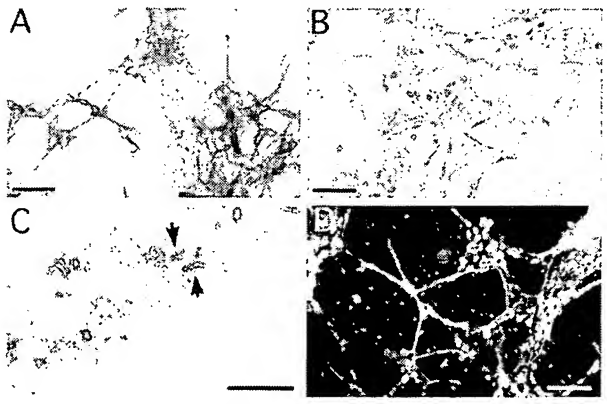

FIG. 1. *In vitro* uptake of β-gal–TTC hybrid protein and control β-gal by 1009 neural cells. Eight days after the start of retinoic treatment, differentiated 1009 cells were incubated at 37°C for 2 hr with 5 μg/ml of each protein and fixed. X-Gal staining was performed after incubation with β-gal–TTC (*A*) or β-gal (*B*). Strong labeling was specifically detected in neural cells. (*C*) Electron micrograph of X-Gal-stained neural cell axon showing intracellular localization of the fusion protein: arrows indicate the precipitate probably associated with filaments. After β-gal–TTC incubation, immunodetection was performed with anti-neurofilament and anti-TTC antibodies (*D*): colabeling showing that β-gal–TTC was only uptaken in neuronal cells. [Bars = 100 μm (*A*, *B*, and *D*) and 1 μm (*C*).]

protein had been internalized. The intracellular localization of the hybrid protein was further confirmed by electron microscopic analysis of X-Gal-stained cells (Fig. 1*C*). Furthermore, the enzymatic activity observed in axons seemed to be localized in vesicles associated with filaments (Fig. 1*C*), which is in agreement with previous work on TTC fragment or native tetanus toxin (14, 21, 22). Colabeling with anti-TTC and anti-neurofilament antibodies revealed that β-gal activity colocalized with TTC fragment in neuronal cells (Fig. 1*D*). No glial cells were labeled with either antibody.

Retrograde Transport of the Hybrid Protein *in Vivo*. To study the behavior of the β-gal–TTC protein *in vivo*, we chose to test the hybrid protein in a well-characterized neuronal network, the hypoglossal system. After intramuscular injection of β-gal–TTC protein into the mouse tongue, the distribution of the hybrid protein in the CNS was analyzed by X-Gal staining. Various dilutions of the protein were injected and sequential time points were analyzed to permit protein transport into hypoglossal motoneurons (XII) and its further transneuronal migration into connected second order neurons.

A well-defined profile of large, apparently retrogradely labeled neurons was clearly evident in the hypoglossal structure, analyzed *in toto* at 12 hr postinjection (Fig. 2 *A* and *B*). A strong labeling was also apparent in the hypoglossal nerve (XIIn) of the tongue of the injected mice (Fig. 2 *C* and *D*). At the level of muscle fibers, button structures were observed that might reflect labeling of neuromuscular junctions where the hybrid protein was internalized into nerve axons (Fig. 2*E*). These data demonstrate that the β-gal–TTC hybrid protein can migrate rapidly by retrograde axonal transport as far as motoneuron cell bodies, after prior uptake by nerve terminals in the tongue. This specific uptake and the intra-axonal transport are similar to the properties that have been described for the native toxin (6, 21, 23).

Transport of the hybrid protein was examined in greater detail by analyzing X-Gal-stained brain sections. Motoneurons of the hypoglossal nucleus became labeled rapidly, with 12 hr being the earliest time point examined. Most of the label was confined to neuronal somata, the cell nuclei being unlabeled. The intensity of the labeling depends upon the concentration of the β-gal–TTC protein injected: when 10 μg of protein was injected, only the hypoglossal somata were detected, whereas with 25–50 μg of protein, a fuzzy network of dendrites was visualized; transynaptic transfer was detected with 100 μg of hybrid protein. An identical distribution of label was observed when brain sections were immunostained with an anti-TTC antibody, demonstrating that β-gal and TTC fragment colocalize within cells. Finally, injection of β-gal alone did not result in labeling of the hypoglossal nuclei and therefore confirms that transport of the hybrid protein is TTC-dependent. In our hands, labeling with an anti-TTC antibody was less informative than detection of β-gal activity; for instance, the nerve pathway to the brain could not be visualized by anti-TTC immunostaining (Fig. 2*H*). At 18 hr postinjection, labeling was observed in the hypoglossal nuclei: all motoneuron cell bodies and the most proximal part of their dendrites were very densely stained (Fig. 2 *F–H*). In contrast, no labeling was ever detected in glial cells adjoining XII motoneurons or their axons. Our results are in accordance with others who reported an identical pattern of immunolabeling after injection of the TTC fragment alone (9). Transneuronal

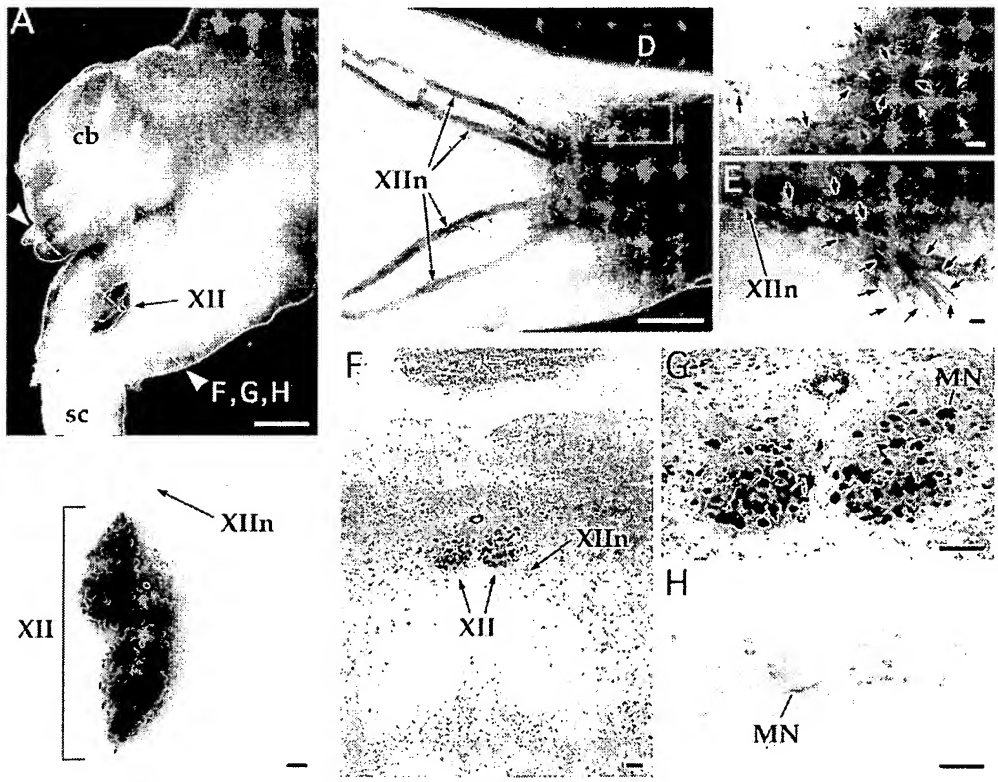

FIG. 2. Retrograde labeling following β-gal–TTC intramuscular injection in the tongue of mice. A total of 100 μg of protein was injected, and observations were made 12 hr (*A, B, C, D,* and *E*) and 18 hr (*F, G,* and *H*) postinjection. *In toto* X-Gal staining on brain clearly showed the hypoglossal structure (XII) with its two nuclei retrogradely labeled (*A* and *B*). The hypoglossal nerve (XIIn) was also intensively stained (*C*), as was its arborization (*E*, see arrows). Putative neuromuscular junctions (*D*) with button-like structure (black arrows) and the terminal arborization of axons (white arrows) are visible. (*F* and *G* with different magnification) Histological analysis of brain slices after X-Gal staining. The hybrid protein is localized in XII motoneurons cytoplasm. (*H*) Immunodetection with anti-TTC antibody showing a colocalization with the β-gal activity. [Bars = 1 mm (*A* and *C*), 100 μm (*B* and *D–H*).] cb, Cerebellum; sc, spinal cord; MN, motoneuron.

transfer is detectable after 24 hr. An additional 24 hr and beyond did not yield a different staining.

Transneuronal Transport of the Hybrid Protein. Second-order interneurons, as well as higher-order neurons that synapse with the hypoglossal motoneurons, have been extensively analyzed using conventional markers, such as the wheat germ agglutinin–horseradish peroxidase complex or neurotropic viruses such as α-herpes (24) and rhabdoviruses (25). An exhaustive compilation of regions in the brain that synaptically connect to the hypoglossal nucleus has also been described (25). In our study, the distribution of the β-gal–TTC fusion depended on the initial concentration of protein injected into the muscle and the time allowed for transport after injection. Up to 24 hr postinjection, labeling was restricted to the hypoglossal nuclei. After 24 hr, the distribution of second-order transneuronally labeled cells in various regions of the brain was consistent and reproducible; however, we have not yet analyzed higher-order connections, such as in cortical areas. Even at longer time points (e.g., 48 hr), labeling of the hypoglossal nucleus remained constant. At higher magnification, a discrete and localized staining of second-order neurons was observed, suggesting that the hybrid protein had been targeted to vesicles within cell somata, synapses, and axons. A similar patchy distribution was previously described for tetanus toxin and TTC fragment alone (14, 21, 22).

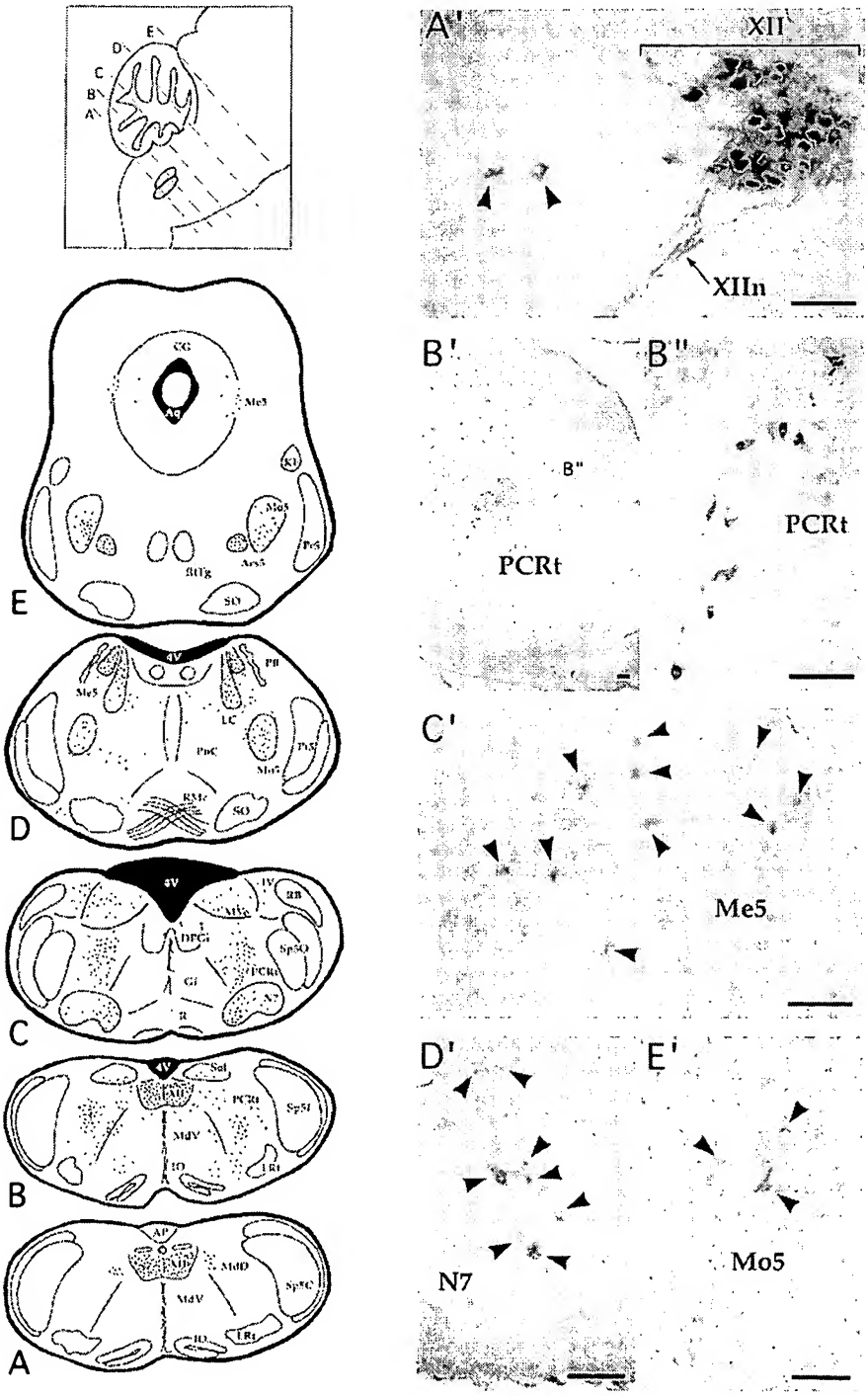

FIG. 3. Transneuronal labeling following intramuscular injection of β-gal–TTC into the mouse tongue. A total of 100 μg of protein was injected, and observations were made 24–48 hr postinjection. (*A–E*) Distribution of β-gal-positive neurons in different brainstem sections, also summarized in Table 1. One dot represents one labeled neuron; six animals were analyzed. (*A′–E′*) Examples of labeled neurons: labeling in medullary reticular dorsal (MdD) area (*A′*), arrows showing second order stained neurons; labeling in parvicellular reticular nucleus (PCRt) area (*B′* and *B″* with different magnification) showing a β-gal-positive column of neurons; labeling in Me5 area (*C′*), arrows show second-order stained neurons; labeling in N7 (*D′*) and Mo5 areas (*E′*), arrows show β-gal-positive neurons. [Bars = 100 μm (*A′*, *B′*, *B″*, *D′*, and *E′*) and 50 μm (*C′*).] AP, area postrema; Aq, Aqueduct (Sylvius); 4V, 4th ventricule; Gi, gigantocellular reticular nucleus (nu); IO, inferior olive; KF, Kölliker–Fuse nu; LRt, lateral reticular nu; PB, parabrachial nu; Pr5, principal sensory trigeminal nu; RtTg, reticulotegmental nu pons; SO, superior olive; Sp5C, spinal trigeminal nu, caudal part; Sp5I, spinal trigeminal nu, interpolar part; Sp5O, spinal trigeminal nu, oral part; see Table 1 for other abbreviations.

Intense transneuronal labeling was detected in the lateral reticular formation (LRF), where medullary reticular neurons have been reported to form numerous projections onto the hypoglossal nucleus (26, 27). β-Gal activity was detected bilaterally in these sections. Labeled LRF projections formed a continuous column along the rostrocaudal axis, beginning lateral to the hypoglossal nucleus, with a few neurons being preferentially stained in the medullary reticular dorsal (MdD) and the medullary reticular ventral (MdV) nuclei (Fig. 3 *A*, *B*, and *A'*). This column extends rostrally through the medulla, with neurons more intensely labeled in the parvicellular reticular nucleus (PCRt, caudal and rostral in Fig. 3 *B*, *C*, and *B'*). After 48 hr, cells in MdD and PCRt were more intensely stained. A second bilateral distribution of medullary neurons projecting to the hypoglossal nucleus was detected in the solitary nucleus (Sol in Fig. 3*B*), but the labeling was less intense than in the reticular formation, presumably because relatively few cells of the solitary nucleus project onto the hypoglossal nucleus (26). However, no labeling was found in the spinal trigeminal nucleus (Sp5), which has also been shown to project onto the hypoglossal nucleus (26). Transynaptic transport of the β-gal–TTC protein was also detected in the pontine reticular nucleus caudal (PnC), the locus coeruleus (LC), the medial vestibular nucleus (MVe), and in a few cells of the inferior vestibular nucleus (IV). These cell groups are known to project onto the hypoglossal nucleus (25), but their labeling was weak, probably because of the greater length of their axons (Fig. 3 *C*, *D*, and *E*). We have observed a few labeled cells in the dorsal paragigantocellular nucleus (DPGi), the magnocellular nucleus caudal (RMc), and the caudal raphe nucleus (R) (Fig. 3 *C*, *D*, and *E*); their connections to the hypoglossal nucleus have also been reported (25). Finally, labeled neurons were detected bilaterally in midbrain projections, such as those of the mesencephalic trigeminal nucleus (Me5 in Fig. 3 *D*, *E*, and *C'*), and a few neurons were stained in the mesencephalic central gray region (CG in Fig. 3*E*). These latter nuclei have been typed as putative third order cell groups related to the hypoglossal nucleus (25).

Neurons in the motor trigeminal nucleus (Mo5 in Fig. 3 *D*, *E*, and *E'*) and the accessory trigeminal tract (Acs5 in Fig. 3*E*) were also labeled, along with a population of neurons in the facial nucleus (N7 in Fig. 3 *C* and *D'*). However, interpretation of this labeling is more ambiguous because it is known that motoneurons in these nuclei also innervate other parts of the muscular tissue, and diffusion of the hybrid protein might have occurred at the point of injection. Conversely, these nuclei may have also projected to the tongue musculature via nerve XII, since neurons in N7 have been reported to receive direct hypoglossal nerve input (28). This latter explanation is consistent with the fact that labeling in these nuclei was detected only after 24 hr; however, this point was not further investigated.

Altogether, the data summarized in Table 1 and Fig. 3 clearly establish transneuronal transport of the β-gal–TTC fusion protein from the hypoglossal neurons into several connected regions of the brainstem.

DISCUSSION

In this study, we show that a β-gal–TTC hybrid protein retains the biological activities of both proteins *in vivo*. Therefore, the hybrid protein can undergo retrograde and transneuronal transport through a chain of interconnected neurons, as traced by its enzymatic activity. Our results are consistent with those of others who used chemically conjugated TTC, or TTC fused to other proteins (12–15). In these *in vitro* analyses, the activity of the conjugated or hybrid proteins was likewise retained or only weakly diminished. Depending on the nature of the TTC fusion partner, different types of potential applications can be envisioned. For example, one might be able to deliver a biologically active protein into the CNS for therapeutic purposes. Such hybrid genes could also be used to analyze and map synaptically connected neurons if reporters such as *lacZ* or the green fluorescent protein (*GFP*; ref. 29) gene were fused to TTC.

The retrograde transport of the hybrid protein is best demonstrated by the data in Fig. 2. When injected into a muscle, β-gal activity rapidly localized to the somata of motoneurons that innervate the muscle. The arborization of the whole nerve, axon, somata, and dendrites can easily be visualized. However, in comparison to the neurotropic viruses, the extent of retrograde transneuronal transport of the hybrid protein from the hypoglossal neurons indicates that only a subset of interconnected neurons is detected, although most areas containing second-order interneurons have been identified by the β-gal–TTC marker. Transneuronal uptake is mostly restricted to second-order neurons. In such experiments, when a limited amount of a neuronal tracer is injected into a muscle or cell, only a fraction will be transported through a synapse, thereby imposing an experimental constraint on its detection. Presently, the most efficient method, in terms of the extent of transport, relies on neurotropic viruses. Examples include: α-herpes viruses, such as herpes simplex type 1, pseudorabies virus, and rhabdoviruses (24, 25). Viral methods are very sensitive because each time a virus infects a new cell, it replicates, thereby amplifying the signal and permitting visualization of higher order neurons in a chain. Ultimately, however, one wants to map a neuronal network in an *in vivo* situation such as a transgenic animal (see below). Here, the disadvantage of viral labeling is its potential toxicity. Most viruses are not innocuous for the neural cell, and their replication induces a cellular response and sometimes cell degeneration (24). Furthermore, depending on experimental conditions, budding of the virus can occur leading to its spread into adjoining cells and tissues.

Differences in mechanisms of transneuronal migration could also account for the restricted number of neurons labeled by β-gal–TTC. Matteoli *et al.* (22) have provided strong evidence that the intact tetanus toxin crosses the synapses by parasitizing the physiological process of synaptic vesicle recycling at the nerve terminal. The toxin probably binds to the inner surface of a synaptic vesicle during the time the lumen is exposed to the external medium. Vesicle endocytosis would then presumably provide the mechanism for internalization of the toxin. Because

Table 1. Transneuronal transport of the *lacZ*-TTC fusion from the XII nerve: labeling of different cells types in the central nervous system

Cell groups	12–18 hr	24–48 hr
First order neurons		
First category		
XII, hypoglossal motoneurons	++	+++
Second category		
N7, facial nu	–	++
Mo5, motor trigeminal nu	–	++
Acs5, accessory trigeminal nu	–	+
Second order cell groups		
MdD, medullary reticular nu, dorsal	–	++
MdV, medullary reticular nu, ventral	–	+/–
PCRt, parvicellular reticular nu, caudal	–	++
PCRt, parvicellular reticular nu, rostral	–	++
Sol, solitary tract nu	–	+
DPGi, dorsal paragigantocellullar nu	–	+/–
PnC, pontine reticular nu, caudal	–	+
RMc, magnocellular reticular nu	–	+/–
R, caudal raphe nu	–	+/–
MVe, medial vestibular nu	–	+
IV, inferior vestibular nu	–	+/–
LC, locus coeruleus	–	+
Me5, mesencephalic trigeminal nu (*)	–	+
CG, mesenphalic central gray (*)	–	+/–

(*), Second-order cell groups that also contain putative third order neurons (see text); –, no labeling; + to +++, increased density of label; +/– weak labeling. Sixteen animals were analyzed for the 12- to 18-hr postinjection data; 6 animals were analyzed for the 24- to 48-hr postinjection data. nu, Nucleus.

the TTC fragment is known to mimic the migration of the toxin *in vivo*, it could therefore direct the fusion protein along a similar transynaptic pathway. If this hypothesis is confirmed, it would strongly suggest that synaptic activity is required for the transneuronal transport of β-gal–TTC. Therefore, only active neuronal circuits would be detected by the hybrid protein. The possible dependence of β-gal–TTC on synaptic vesicle exocytosis and endocytosis could be further investigated, since techniques are now available to record synaptic activity in neural networks *in vitro* (30). In contrast, the transneuronal pathway of neurotropic viruses has not yet been elucidated and could be fundamentally different, involving virus budding in the vicinity of a synapse. Finally, the transneuronal transport of the hybrid protein might depend on a synaptic specificity, although the tetanus toxin is not known to display any (7, 23); it is therefore likely that a virus would cross different or inactive synapses. In summary, the restricted spectrum of interneuronal transport, in addition to its nontoxicity, make the β-gal–TTC hybrid protein a novel and powerful tool for analysis of neural pathways.

In our view, the one advantage of the fusion gene that we describe for neuronal mapping is that it derives from a single genetic entity that is amenable to genetic manipulation and engineering. Several years ago, a technique based on homologous recombination in embryonic stem cells was developed to specifically replace genes in the mouse (31, 32). This method generates a null mutation in the substituted gene, although in a slightly modified strategy, a dicistronic messenger RNA can also be produced (33, 34). When a reporter gene, such as *E. coli lacZ*, is used as the substituting gene, this technique provides a means of marking the mutated cells so that they can be followed during embryogenesis. Thus, this technique greatly simplifies the analysis of both the heterozygote expression of the targeted gene as well as the phenotype of null (homozygous) mutant animals.

Neural cells establish specific and complex networks of interconnected cells. If a gene were mutated in a given neural cell, we would expect this mutation to have an impact on the functions of other, interconnected neural cells. With these considerations in mind, a genetic marker that can diffuse through active synapses would be very useful in analyzing the effect of the mutation. In heterozygous mutant animals, the cells in which the targeted gene is normally transcribed could be identified, as could the synaptically connected cells of a neural network. In a homozygous animal, the impact of the mutation on the establishment or activity of the neural network could be determined. The feasibility of such an *in vivo* approach depends critically on the efficiency of synaptic transfer of the fusion protein, as well as its stability and cellular localization. We are currently generating various transgenic animals based on this type of gene fusion to evaluate the application of this technique to the study of CNS development.

Another extension of our approach is to gene therapy applied to the CNS. We have shown that a nontoxic, enzyme–vector conjugate is taken up by axon terminals and conveyed retrogradely to brainstem motoneurons. A selective retrograde transynaptic mechanism subsequently transports the hybrid protein into second-order connected neurons. Such a pathway, which bypasses the blood–brain barrier, could be exploited to deliver macromolecules to the CNS. In fact, pathogenic agents such as tetanus toxin and neurotropic viruses are similarly taken up by nerve endings, internalized and retrogradely transported to the nerve cell somata. In such a scenario, the *lacZ* reporter would be replaced by a gene encoding a protein that provides a necessary or interesting activity and/or function. For example, the human CuZn superoxide dismutase (SOD-1) and the human enzyme β-*N*-acetylhexosaminidase A have been fused or chemically coupled to the TTC fragment (13, 16), and their uptake by neurons *in vitro* was considerably increased and their enzymatic functions partially conserved. Combined with the *in vivo* experiments described here using β-gal–TTC, a gene therapy approach based on TTC hybrid proteins appears to be a feasible method of delivering a biological function to the CNS. However, ways have to be found to target the TTC hybrid proteins, which are likely to be sequestrated into vesicles, to the appropriate subcellular compartment. Such a therapeutic strategy could be particularly useful for treating neurodegenerative and motoneuron diseases, such as amyotrophy lateral sclerosis (35), spinal muscular atrophies (36, 37), or neurodegenerative lysosomal storage diseases (38, 39). Injection into selected muscles, even *in utero*, could help to specifically target the appropriate neurons. In addition, such an approach would avoid the secondary, and potentially toxic, effects associated with the use of defective viruses to deliver a gene (40, 41).

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Exhibit 2

LEXSEE 435 F.3D 1366

ENERGIZER HOLDINGS, INC. and EVEREADY BATTERY COMPANY, INC., Appellants, v. INTERNATIONAL TRADE COMMISSION, Appellee, and PT INTERNATIONAL CHEMICAL INDUSTRIAL CO. LTD., Intervenor, and GOLDEN POWER INDUSTRIES, LTD., GUANGDONG CHAOAN ZHENGLONG ENTERPRISE CO., LTD., GUANGZHOU TIGER HEAD BATTERY GROUP CO., LTD., FUJIAN NANPING NANFU BATTERY CO., LTD., HI-WATT BATTERY INDUSTRY CO., LTD., NINGBO BAOWANG BATTERY CO., LTD., SICHUAN CHANGHONG ELECTRIC CO., LTD., ZHEJIANG 3-TURN BATTERY CO., LTD., and ZHONGYIN (NINGBO) BATTERY CO., LTD., Intervenors.

05-1018

UNITED STATES COURT OF APPEALS FOR THE FEDERAL CIRCUIT

435 F.3d 1366; 2006 U.S. App. LEXIS 1760; 77 U.S.P.Q.2D (BNA) 1625

January 25, 2006, Decided

PRIOR HISTORY: [**1] Appealed from: United States International Trade Commission.
In re Certain Zero-Mercury-Added Alkaline Batteries, 2004 ITC LEXIS 789 (Int'l Trade Comm'n, Oct. 1, 2004)

DISPOSITION: REVERSED and REMANDED.

COUNSEL: Randall G. Litton, Price, Heneveld, Cooper, DeWitt & Litton, LLP, of Grand Rapids, Michigan, argued for appellants. With him on the brief were Eugene J. Rath III and Matthew J. Gipson; Of counsel on the brief were V. James Adduci II and Maureen F. Browne, Adduci, Mastriani & Schaumberg, L.L.P., of Washington, DC. Of counsel were Michael L. Doane, David F. Nickel, Sarah E. Hamblin and S. Alex Lasher.

Wayne W. Herrington, Attorney, Office of the General Counsel, United States International Trade Commission, of Washington, DC, argued for appellee. With him on the brief was James M. Lyons, General Counsel. Of counsel was Neal J. Reynolds, Attorney.

Kent R. Stevens, of Washington, DC, argued for intervenor PT International Chemical Industrial Co., Ltd.

Steven P. Hollman, Hogan & Hartson L.L.P., of Washington, DC, argued for intervenors Golden Power Industries, Ltd., et al. With him on the brief were Christopher T. Handman, Susan M. Cook and Jessica L. Ellsworth; Of counsel on the brief were William E. Thomson, Jr., Wei-Ning Yang, Yoncha L. Kundupoglu and Olga Berson, of Los Angeles, [**2] California. Of counsel was Robert B. Wolinsky.

JUDGES: Before NEWMAN, Circuit Judge, ARCHER, Senior Circuit Judge, and SCHALL, Circuit Judge.

OPINION BY: NEWMAN

OPINION

[*1367] NEWMAN, Circuit Judge.

Energizer Holdings, Inc. and Eveready Battery Company, Inc. (collectively [*1368] "EBC") appeal the ruling of the International Trade Commission in an action under section 337 of the Tariff Act of 1930 as amended (19 U.S.C. § 1337), holding all of the claims of EBC's United States Patent No. 5,464,709 ("the '709 Patent") invalid for failure to comply with 35 U.S.C. § 112 P2. [1] We reverse the holding of invalidity and remand for further proceedings.

1 In re Certain Zero-Mercury-Added Alkaline Batteries, Parts Thereof, and Products Containing Same, Inv. No. 337-TA-493 (Int'l Trade Comm'n, June 2, 2004 (Initial Determination); October 1, 2004 (Final Determination)).

The Invention

The '709 patent is for an electrolytic alkaline battery cell that is substantially free of mercury. Alkaline battery

cells typically contain an electrolyte such as potassium hydroxide, a metal oxide cathode such as manganese dioxide, and a zinc anode. [**3] A detrimental characteristic of alkaline cells has been corrosion of the zinc after partial discharge, producing hydrogen gas which exerts internal pressure, causing the cell to leak. A widely used corrosion inhibitor in such cells is mercury, which amalgamates with the zinc and inhibits hydrogen formation. Mercury, however, is an environmental pollutant, and extensive effort has been devoted to reducing or eliminating the mercury content in alkaline batteries.

The '709 patent describes the discovery that a cause of gas-producing corrosion is the presence of trace impurities in the zinc used in the anode, and that upon identification and elimination of these impurities, the addition of mercury can be eliminated or substantially reduced. The electrolytic cells at issue are described as "zero-mercury-added" batteries. EBC charged the respondents/Intervenors with violation of 19 U.S.C. § 1337 based on their importation into the United States, sale for importation, and sale within the United States, of batteries asserted to infringe the '709 patent.

In the Commission proceedings, the Administrative Law Judge construed the patent claims and held them valid and [**4] infringed. The Commission rejected the ALJ's claim construction and held all of the claims invalid for indefiniteness under 35 U.S.C. § 112 P2, on the ground that the claim term "said zinc anode" lacks antecedent basis in the claim, and that the claims are unclear or ambiguous. EBC challenges this ruling, arguing that the meaning of "said zinc anode" and other usages in the claims, viewed in light of the specification, would be readily understood by persons of ordinary skill in the field of the invention, and that the absence of antecedent basis is not an invalidating flaw.

Standard of Review

We review the Commission's decision on the criteria established by 19 U.S.C. § 1337(c) and the Administrative Procedure Act. In accordance with the APA, agency factual findings are sustained unless they are arbitrary, capricious, or unsupported by substantial evidence, and agency rulings of law are reviewed for correctness. 5 U.S.C. § 706(2)(E). See Jazz Photo Corp. v. Int'l Trade Comm'n, 264 F.3d 1094, 1099 (Fed. Cir. 2001) (applying the APA to review of decisions of the International Trade [**5] Commission).

An analysis of claim indefiniteness under § 112 P2 is "inextricably intertwined with claim construction." Atmel Corp. v. Information Storage Devices, Inc., 198 F.3d 1374, 1379 (Fed. Cir. 1999). See Datamize v. Plumtree Software, Inc., 417 F.3d 1342, 1347-48 [*1369] (Fed. Cir. 2005) ("'By finding claims indefinite only if reasonable efforts at claim construction prove futile, we accord respect to the statutory presumption of validity and we protect the inventive contribution of patentees, even when the drafting of their patents has been less than ideal.'") (quoting Exxon Research & Eng'g Co. v United States, 265 F.3d 1371, 1375 (Fed Cir 2001)); Oakley, Inc. v. Sunglass Hut Int'l, 316 F.3d 1331, 1340-41 (Fed. Cir. 2003) (determination of claim definiteness "requires a construction of the claims according to the familiar canons of claim construction"). Accordingly, we give de novo review to the Commission's ruling of patent invalidity for claim indefiniteness.

35 U.S.C. § 112 P2 requires that the patent specification shall "conclude with one or more claims particularly pointing out [**6] and distinctly claiming the subject matter which the applicant regards as his invention." This provision both facilitates examination during the patent application stage, and upon grant serves to notify the public of what is patented. The reviewing tribunal must determine whether a person experienced in the field of the invention would understand the scope of the claim when read in light of the specification. See Howmedica Osteonics Corp. v. Tranquil Prospects, Ltd., 401 F.3d 1367, 1371 (Fed. Cir. 2005) (claim not indefinite due to ambiguity when meaning readily ascertained from the description in the specification); Personalized Media Communs., L.L.C. v. ITC, 161 F.3d 696, 705 (Fed. Cir. 1998). See generally Phillips v. AWH Corp., 415 F.3d 1303, 1313 (Fed. Cir. 2005) (en *banc*) (claims are construed in the context of the specification and prosecution history, as they would be understood by persons in the same field of endeavor).

The Commission's Decision

The Commission held invalid independent claim 1 and dependent claims 2-7, for the claims all contain the usage to which the Commission objected. [**7] Claim 1 is as follows, with emphasis added to the term that was the focus of the Commission's decision:

> 1. An electrochemical cell comprising an alkaline electrolyte, a cathode comprising manganese dioxide as an active cathode component, and an anode gel comprised of zinc as the active anode component, wherein the cell contains less than 50 parts of mercury per million parts by weight of the cell and **said zinc anode** has a gel expansion of less than 25% after being discharged for 161 minutes to 15% depth of discharge at 2.88A.

The Commission held the claim invalid because "said zinc anode" does not have an antecedent basis, the Commission explaining that "the term 'zinc anode' does

not appear in the claim prior to the term 'said zinc'" anode. Final Det. at 22. The Commission also held that even if "said zinc anode" is construed to have as antecedent the "anode gel comprised of zinc as the active anode component" recited earlier in the claim, the claim would still be indefinite because, as written, it requires that the anode of every cell has been "discharged for 161 minutes to 15% depth of discharge at 2.88A." The Commission reasoned that since it appears that [**8] these discharge parameters are intended to apply only to a test cell, and not that every cell must be discharged, this unclear and ambiguous claim drafting renders the claim "indefinite as a matter of law." The Commission also held that EBC's proffer of alternative constructions of "said zinc anode" was an admission of indefiniteness.

DISCUSSION

The Commission held the claim invalid because the term "said zinc anode" lacked [*1370] an antecedent basis, and because of the claim's imprecise statement of the role of the test parameters. EBC argues that these flaws do not render the claim "insolubly ambiguous," in the words of Marley Mouldings, Ltd. v Mikron Industries, 417 F.3d 1356, 1361 (Fed. Cir. 2005), which held that when a claim "is not insolubly ambiguous, it is not invalid for indefiniteness." See also Bancorp Servs., L.L.C. v Hartford Life Ins. Co., 359 F.3d 1367, 1371 (Fed. Cir. 2004) ("We have held that a claim is not indefinite merely because it poses a difficult issue of claim construction; if the claim is subject to construction, i.e., it is not insolubly ambiguous, it is not invalid for indefiniteness.").

EBC argues that a person of ordinary [**9] skill in this field would readily understand the claim despite imperfect drafting, for the specification makes clear that the test parameters included in the claim do not mean that every cell must be discharged for 161 minutes. EBC points out that the '709 patent is directed to a standard electrolytic alkaline cell, and that the specification clearly states that the purpose of the test is to identify zinc anode material used in the invention.

Claim definiteness is analyzed "not in a vacuum, but always in light of the teachings of the prior art and of the particular application disclosure as it would be interpreted by one possessing the ordinary level of skill in the pertinent art." In re Moore, 58 C.C.P.A. 1042, 439 F.2d 1232, 1235 (CCPA 1971). The definiteness inquiry "focuses on whether those skilled in the art would understand the scope of the claim when the claim is read in light of the rest of the specification." Union Pac. Res. Co. v. Chesapeake Energy Corp., 236 F.3d 684, 692 (Fed. Cir. 2001). Although neither the Commission nor the courts can rewrite claims to correct material errors, the issue here is not correction of error, but understanding [**10] of what the claim covers. When the meaning of the claim would reasonably be understood by persons of ordinary skill when read in light of the specification, the claim is not subject to invalidity upon departure from the protocol of "antecedent basis."

The requirement of antecedent basis is a rule of patent drafting, administered during patent examination. The Manual of Patent Examining Procedure states that "obviously, however, the failure to provide explicit antecedent basis for terms does not always render a claim indefinite." MPEP § 2173.05(e) (8th ed. Rev. 2, May, 2004). In Slimfold Manufacturing Co. v. Kinkead Industries, Inc., 810 F.2d 1113, 1117 (Fed. Cir. 1987) the court held that "the missing antecedent clause, the absence of which was not observed by the examiner of the original patent or by Kinkead in its reissue protest documents, did not fail to inform the public during the life of the ['274] patent of the limits of the monopoly asserted." The Slimfold court held that addition of the missing antecedent basis during reissue was not a substantive change.

Whether this claim, despite lack of explicit antecedent basis for "said zinc anode," nonetheless [**11] has a reasonably ascertainable meaning must be decided in context. In prosecuting the '709 patent, the examiner made several objections to the claims, but the claims were not rejected or objected to on the ground of lack of antecedent basis. In Bose Corp. v. JBL, Inc., 274 F.3d 1354, 1359 (Fed. Cir. 2001) the court held that despite the absence of explicit antecedent basis, "If the scope of a claim would be reasonably ascertainable by those skilled in the art, [*1371] then the claim is not indefinite." Moreover, we noted in Slimfold that an antecedent basis can be present by implication. Slimfold, 810 F.2d at 1116. See Cross Medical Products v. Medtronic Sofamor Danek, 424 F.3d 1293, 1319 (Fed. Cir. 2005).

Neither the Commission nor the Intervenors argued that they did not understand the intended scope because of the absence of an antecedent. The Commission erred in holding that the need to construe a claim, or the proffer of alternative constructions, renders the claim indefinite. A claim that is amenable to construction is not invalid on the ground of indefiniteness. In Exxon Research & Engineering, 265 F.3d at 1375, [**12] the court stated that "if the meaning of the claim is discernible, even though the task may be formidable and the conclusion may be one over which reasonable persons will disagree, we have held the claim sufficiently clear to avoid invalidity on indefiniteness grounds." See also Novo Indus., L.P. v. Micro Molds Corp., 350 F.3d 1348, 1353 (Fed. Cir. 2003) (determining whether claim is "amenable to construction"); Honeywell Int'l, Inc. v. ITC, 341 F.3d 1332, 1338 (Fed. Cir. 2003) (a claim is not indefinite because it is hard to construe). Here, it is apparent that the claim can be construed. In that regard, we conclude

that "anode gel" is by implication the antecedent basis for "said zinc anode." The Commission's holding of invalidity on the ground of indefiniteness is reversed.

No other issues of validity and infringement are before us on appeal. The case is remanded for further proceedings.

REVERSED and REMANDED

Exhibit 3

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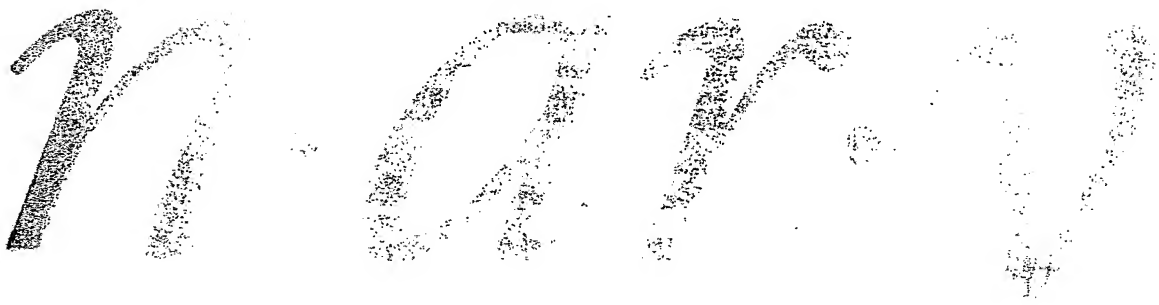
THIRD EDITION



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i·dol·a·ter or **i·dol·a·tor** (ī-dŏl′ə-tər) *n.* **1.** One who worships idols. **2.** One who blindly or excessively admires or adores another. [ME *idolatre* < OFr. < Lat. *idōlolatrēs* < Gk. *eidōlolatrēs* : *eidōlon*, idol; see IDOL + *-latrēs*, worshiper.]

i·dol·a·trous (ī-dŏl′ə-trəs) *adj.* **1.** Of or constituting idolatry. **2.** Given to blind or excessive devotion to something. — **i·dol′a·trous·ly** *adv.* — **i·dol′a·trous·ness** *n.*

i·dol·a·try (ī-dŏl′ə-trē) *n., pl.* **-tries. 1.** Worship of idols. **2.** Blind or excessive devotion to something. [ME *idolatrie* < OFr. < Lat. *īdōlolatria* < Gk. *eidōlolatria* : *eidōlon*, idol; see IDOL + *latreia*, service.]

i·dol·ize (īd′l-īz′) *tr.v.* **-ized, -iz·ing, -iz·es. 1.** To regard with idolatrous devotion. See Syns at revere[1]. **2.** To worship as an idol. — **i′dol·i·za′tion** (-ī-zā′shən) *n.* — **i′dol·iz′er** *n.*

IDP *abbr.* **1.** *Comp. Sci.* Integrated data processing. **2.** International driving permit.

i·dyll also **i·dyl** (īd′l) *n.* **1.a.** A short poem or prose piece depicting a rural or pastoral scene, usu. in idealized terms. **b.** A narrative poem treating an epic or romantic theme. **2.** A scene or an event of a simple and tranquil nature. **3.a.** A carefree episode or experience: *a summer idyll in France.* **b.** A romantic interlude. [Lat. *idyllium* < Gk. *eidullion*, dim. of *eidos*, form, figure. See weid-*.] — **i·dyl′li·cal·ly** *adv.*

i·dyl·lic (ī-dĭl′ĭk) *adj.* **1.** Of or having the nature of an idyll. **2.** Simple and carefree: *an idyllic vacation.*

i·dyl·list (īd′l-ĭst) *n.* A writer of idylls.

IE or **I.E.** *abbr.* **1.** Industrial engineer. **2.** Industrial engineering. **3.** Indo-European.

i.e. *abbr. Lat.* id est (that is).

-ie *suff.* Var. of **-y**[3].

Ie·per (yā′pər) also **Y·pres** (ē′prə). A city of W Belgium near the French border S of Ostend; site of three World War I battles (1914, 1915, and 1917). Pop. 21,200.

if (ĭf) *conj.* **1.a.** In the event that: *If I were to go, I would be late.* **b.** Granting that: *If that is true, what next?* **c.** On the condition that: *She will sing only if paid.* **2.** Although possibly; even though: *a handsome if useless trinket.* **3.** Whether: *Ask if he plans to come.* **4.** Used to introduce an exclamatory clause, indicating a wish: *If they had only come!* — *n.* A possibility, condition, or stipulation. [ME < OE *gif*. See i-*.]

Usage Note: In informal writing both *if* and *whether* are standard in their use to introduce a clause indicating uncertainty after a verb such as *ask, doubt, know, learn,* or *see*: *We shall soon learn whether* (or *if*) *it is true.* In such contexts, however, the use of *if* can sometimes create ambiguities. Depending on the intended meaning, the sentence *Let her know if she is invited* might be better paraphrased as *Let her know whether she is invited* or *If she is invited, let her know.* • In conditional sentences the clause introduced by *if* may contain either a past subjunctive verb (*if I were going*) or an indicative verb (*if I was going*), depending on the intended meaning. According to the traditional rule, the subjunctive should be used to describe an occurrence that is presupposed to be contrary to fact, as in *if I were ten years younger.* The main verb of such a sentence must then contain the modal verb *would* or (less frequently) *should*: *If I were you, I should* (or *would*) *buy new shirts.* When the situation described by the *if* clause is not presupposed to be false, however, that clause must contain an indicative verb, and the choice of verb in the main clause will depend on the intended meaning: *If Kevin was out all day, then he couldn't call back.* • Again according to the traditional rule, the subjunctive is not correctly used following verbs such as *ask* or *wonder* in *if* clauses that express indirect questions, even if the content of the question is presumed to be contrary to fact: *We wondered if dinner was* (not *were*) *included in the room price.* See Usage Notes at should, wish.

IF or **I.F.** *abbr.* Intermediate frequency.

I·fe (ē′fā). A city of SW Nigeria E of Ibadan; center of a powerful Yoruba kingdom until the late 17th cent. Pop. 209,100.

If·fy (ĭf′ē) *adj.* **-fi·er, -fi·est.** *Informal.* Doubtful; uncertain.

If·ni (ĕf′nē). A former Spanish possession (1860–1969) on the Atlantic coast of SW Morocco.

IFO *abbr.* Identified flying object.

I formation *n. Football.* An alignment of the offensive team in which all the backs line up in single file behind the center.

IFR *abbr.* Instrument flight rules.

-ify *suff.* Var. of **-fy.**

Ig *abbr.* Immunoglobulin.

IG or **I.G.** *abbr.* Inspector general.

igg (ĭg) *tr.v.* **igged, igg·ing, iggs.** *Northern U.S.* To ignore.

Regional Note: Igg, a shortened form of *ignore*, seems to have come into American speech from jive, the special jargon of Black jazz musicians in the 1930's. Its use has spread from the musicians' jargon into the Black communities of Northern U.S. cities. Such reduction of a word to its initial syllable is a common source of slang or informal words.

Ig·bo (ĭg′bō) *n.* Var. of **Ibo.**

ig·loo (ĭg′lōō) *n., pl.* **-loos. 1.** An Eskimo dwelling, esp. a dome-shaped dwelling built of blocks of packed snow. **2.** A dome-shaped structure or building. [Canadian Eskimo *iglu*, house.]

ign. *abbr.* Ignition.

Ig·na·tius (ĭg-nā′shəs), Saint. d. c. A.D. 110. Bishop of Antioch who was martyred during the reign of Trajan.

Ignatius of Loy·o·la (loi-ō′lə), Saint. 1491–1556. Spanish ecclesiastic who founded the Jesuits.

ig·ne·ous (ĭg′nē-əs) *adj.* **1.** Of, relating to, or characteristic of fire. **2.** *Geol.* **a.** Formed by solidification from a molten state. Used of rocks. **b.** Of or relating to rock so formed; pyrogenic. [< Lat. *igneus* < *ignis*, fire.]

ig·nim·brite (ĭg′nĭm-brīt′) *n.* A volcanic rock formed by the welding together of tuffs from an explosive volcanic eruption. [Lat. *ignis*, fire + *imber, imbr-*, rain + **-ITE**[1].]

ig·nis fat·u·us (ĭg′nĭs făch′ōō-əs) *n., pl.* **ig·nes fat·u·i** (ĭg′nēz făch′ōō-ī′). **1.** A phosphorescent light that hovers or flits over swampy ground at night, possibly from spontaneous combustion of gases emitted by rotting organic matter. **2.** Something that misleads or deludes; an illusion. [Med.Lat. : Lat. *ignis*, fire + Lat. *fatuus*, foolish.]

ig·nite (ĭg-nīt′) *v.* **-nit·ed, -nit·ing, -nites.** — *tr.* **1.a.** To cause to burn. **b.** To set fire to. **2.** To subject to great heat, esp. to make luminous by heat. **3.** To arouse the passions of; excite: *The insults ignited my anger.* — *intr.* **1.** To begin to burn. **2.** To begin to glow. [LLat. *ignīre, ignīt-* < Lat. *ignis*, fire.] — **ig·nit′a·ble, ig·nit′i·ble** *adj.* — **ig·nit′er, ig·ni′tor** *n.*

ig·ni·tion (ĭg-nĭsh′ən) *n.* **1.** The raising of a substance to its ignition point, as by electric current. **2.a.** An electrical system, typically powered by a battery or magneto, that provides the spark to ignite the fuel mixture in an internal-combustion engine. **b.** A switch that activates this system.

ignition point *n.* The minimum temperature at which a substance will continue to burn without additional external heat.

ig·ni·tron (ĭg-nī′trŏn′, ĭg′nĭ-) *n.* A single-anode mercury-vapor rectifier in which current passes as an arc between an anode and cathode, consisting of liquid mercury, used in power rectification. [IGNI(TE) + **-TRON.**]

ig·no·ble (ĭg-nō′bəl) *adj.* **1.** Not noble in quality, character, or purpose; base or mean. **2.** Not of the nobility; common. [ME, of low birth < OFr. < Lat. *ignōbilis* : *i-, in-*, not; see **IN-**[1] + *nōbilis, gnōbilis*, noble; see **NOBLE.**] — **ig·no·bil′i·ty** (-bĭl′ĭ-tē), **ig·no′ble·ness** *n.* — **ig·no′bly** *adv.*

ig·no·min·i·ous (ĭg′nə-mĭn′ē-əs) *adj.* **1.** Marked by shame or disgrace. **2.** Deserving disgrace or shame; despicable. **3.** Degrading; debasing: *an ignominious task.* — **ig′no·min′i·ous·ly** *adv.* — **ig′no·min′i·ous·ness** *n.*

ig·no·min·y (ĭg′nə-mĭn′ē, -mə-nē) *n., pl.* **-ies. 1.** Great personal dishonor or humiliation. **2.** Shameful or disgraceful action, conduct, or character. [Fr. *ignominie* < OFr. < Lat. *ignōminia* : *i-, in-*, not; see **IN-**[1] + *nōmen, nōmin-*, name, reputation; see nō-men-*.]

ig·no·ra·mus (ĭg′nə-rā′məs) *n., pl.* **-mus·es.** An ignorant person. [Ult. < Lat. *ignōrāmus*, we do not know, first pers. pl. pr.t. of *ignōrāre*, to be ignorant. See **IGNORE.**]

ig·no·rance (ĭg′nər-əns) *n.* The condition of being uneducated, unaware, or uninformed.

ig·no·rant (ĭg′nər-ənt) *adj.* **1.** Lacking education or knowledge. **2.** Showing or arising from a lack of education or knowledge: *an ignorant mistake.* **3.** Unaware or uninformed. [ME *ignoraunt* < OFr. *ignorant* < Lat. *ignōrāns, ignōrant-*, pr.part. of *ignōrāre*, to be ignorant, not to know. See gnō-*.] — **ig′no·rant·ly** *adv.*

ig·nore (ĭg-nôr′, -nōr′) *tr.v.* **-nored, -nor·ing, -nores.** To refuse to pay attention to; disregard. See Regional Note at **igg.** [Fr. *ignorer* < OFr. < Lat. *ignōrāre*. See gnō-*.] — **ig·nor′a·ble** *adj.* — **ig·nor′er** *n.*

I·go·rot (ĭg′ə-rōt′, ē′gə-) *n., pl.* **Igorot** or **-rots. 1.** A member of any of several peoples of the mountains of northern Luzon in the Philippines. **2.** Any of the Austronesian languages of the Igorot.

I·gua·çú also **I·guas·sú** (ē′gwə-sōō′). A river of S Brazil flowing c. 1,199 km (745 mi) to the Paraná R. at the Argentina-Paraguay-Brazil border. Just above the junction it forms **Iguaçú Falls**, a series of cataracts averaging 61 m (200 ft) high.

I·gua·na (ĭ-gwä′nə) *n.* Any of various large tropical American lizards of the family Iguanidae, often having spiny projections along the back. [Sp. < Arawak *iwana*.]

I·guan·o·don (ĭ-gwä′nə-dŏn′) *n.* Any of various large dinosaurs of the genus *Iguanodon*, of the Jurassic and Cretaceous periods. [NLat. *Iguanodon*, genus name : IGUANA + **-ODON.**]

ihp or **I.hp.** *abbr.* Indicated horsepower.

Ih·ram (ē-räm′) *n. Islam.* **1.** The sacred dress of Muslim pilgrims, consisting of two lengths of white cotton. **2.** The sacred state of Muslim pilgrims wearing this dress at a time of great self-denial. [Ar. *iḥrām*, prohibition, ihram < *'aḥrama*, to consecrate.]

IHS *abbr.* Jesus (Greek IHΣOYΣ with S for sigma).

Ijs·sel or **IJs·sel** (ī′səl). A river, c. 113 km (70 mi) of E Netherlands flowing from the Lower Rhine R. to the Ijsselmeer.

Ijs·sel·meer or **IJs·sel·meer** (ī′səl-mâr′, -mār′). A shallow dike-enclosed lake of NW Netherlands; formed from the Zuider Zee by the construction of two dams (completed 1932).

I·ka·ri·a (ē′kä-rē′ä) also **I·car·i·a** (ĭ-kâr′ē-ə, ī-kâr′-). An island of SE Greece in the Aegean Sea W of Samos.

Iguana
Common iguana
Iguana iguana

Iguanodon
Ouranosaurus